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<p>(54) Title: EXPRESSION OF STARCH-BINDING DOMAIN (SBD)</p> <p>(57) Abstract</p> <p>The present invention relates to construction and expression of stable starch-binding domain from maltogenic amylases and related enzymes.</p>		

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Expression of starch-binding domain(SBD)**THE FIELD OF THE INVENTION**

5 The present invention relates to a DNA construct encoding a starch-binding domain being a region in the maltogenic amylase enzyme product produced by *Bacillus stearothermophilus* C599 disclosed in EP patent no. 120,693 (Novo Industri A/S), the starch-binding domain itself, an expression vector comprising
10 the starch-binding domain of the invention, a host cell transformed with said expression vector of the invention, a method for expressing the starch-binding domain of the invention in a *Bacillus* sp. host strain transformed with the expression vector of the invention.

15

BACKGROUND OF THE INVENTION

EP patent no. 120,693 discloses a maltogenic amylase derived from *Bacillus stearothermophilus* C599.

20 Starch-binding domain

In the following "Starch Binding Domain" will be abbreviated as "SBD" and is meant to define all polypeptide sequences or peptide sequences having affinity for binding to starch.

Most known SBDs today are found in CGTases, i.e. cyclodextrin
25 glucanotransferases (E.C. 2.4.1.19), and glucoamylases (E.C. 3.2.1.3). See also Chen et al. (1991), Gene 991, p. 121-126, describing Starch Binding Domain hybrids.

Specifically, a SBD has been found in the commercially available enzyme AMG™ (a glucoamylase) from *Aspergillus niger* (Belshaw
30 et al. (1993), "Specificity of the binding domain of glycoamylase I" Eur. J. Biochem, 211:717-724). Furthermore, a SBD from a CGTase derived from *Bacillus macerans* has also been described.

Dalmia et al. (1995), Biothech. Bioeng., Vol 47, pp. 576-584

describes expression of the starch-binding domain (E-domain) of glucoamylase I from *Aspergillus awamori* and of a CGTase from *Bacillus macerans* in *E.coli*.

Expression of SBDs in *E. coli* is not a true extracellular
5 expression and results in an unsatisfactory yield which is too low for industrial scale production of SBD. Further, *E.coli* expression results in intracellular none-soluble protein aggregate (i.e. inclusion bodies).

Accordingly, there is a need for a method for producing SBDs
10 in a high yield and/or by means of a conventional fermentation technique involving extracellular production of the SBDs which makes the use of SBDs in industrial applications economically feasible.

15 SUMMARY OF THE INVENTION

It is the object of the present invention to provide single SBDs (or isolated SBDs) and to express the single SBDs in a *Bacillus* host cell.

20 Single starch-binding domain (single SBD)

The term "single SBD" may also be referred to as "isolated SBD" or "separate SBD".

In the context of the present invention a "single SBD" includes up to the entire part of the amino acid sequence of a SBD-
25 containing enzyme, e.g. a polysaccharide hydrolyzing enzyme, being essentially free of the catalytic domain, but retaining the SBD unit(s).

Thus, in the context of the invention, the entire catalytic amino acid sequence of a starch degrading enzyme (e.g. a glucoamylase) or other enzymes comprising one or more SBD units is
30 not to be regarded as a single SBD.

Typically a single SBD constitutes one or more SBD units of a polysaccharide hydrolyzing enzyme, one or more SBD units of a

starch binding protein or a protein designed and/or engineered to be capable of binding to starch.

The single SBD is at least as large as the minimum number of amino acids in the amino acid sequence required to bind to starch.

A single SBD may also be an amino acid sequence, in which the binding and catalytic domain are one and the same.

Isolation of a starch-binding domain

In order to isolate the starch-binding domain of e.g. a glucoamylase, several genetic approaches may be used. One method uses restriction enzymes to remove a portion of the gene and then to fuse the remaining gene-vector fragment in frame to obtain a mutated gene that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as Bal31 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene deletion methods result in a mutated gene encoding a shortened gene molecule which may then be evaluated for starch binding ability.

Enzyme-SBD hybrid construction

Once a DNA sequence encoding the starch binding domain has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding an enzyme of interest. The starch-binding domain encoding fragment and the DNA encoding the enzyme of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to provide for expression. Microbial hosts such as *Aspergillus*, e.g., *A. niger* and *A. oryzae*, *Bacillus*, *E. coli* or *S. cerevisiae* are preferred.

In the first aspect the invention relates to an isolated DNA sequence comprising a DNA sequence encoding the E-domain (SEQ ID

NO: 2) of the maltogenic amylase produced by *Bacillus stea-*
rothermophilus C599, without having the enzymatic activity of
the maltogenic amylase produced by *Bacillus stearothermophilus*
C599.

5 The *Bacillus stearothermophilus* C599 is described in EP
120,693.

 In the second aspect the invention relates to a single SBD
polypeptide encoded by a DNA sequence of the invention encoding
a SBD of the invention, in particular the ones shown in SEQ ID
10 NO: 2 (E domain) or SEQ ID NO: 4 (D+E domain).

 In the third aspect the invention relates to a *Bacillus* host
transformed with a vector comprising a DNA sequence coding for a
starch-binding domain of the invention and which is capable of
expressing said sequence.

15 In the forth aspect the invention relates to a *Bacillus* ex-
pression vector which carries an inserted DNA sequence encoding
for a starch-binding domain, in particular an isolated DNA se-
quence of the invention as described above.

 Further, the invention relates to a method of producing a
20 single starch-binding domain polypeptide in a *Bacillus* host
cell, the method comprising the steps of:
- growing under conditions to overproduce starch-binding domains
in a nutrient medium a *Bacillus* host cell which has been trans-
formed with an expression cassette which includes, as operably
25 joined components:

- a) a transcriptional and translational initiation regulatory re-
gion,
- b) a DNA sequence encoding the starch-binding domain polypep-
tide,
- 30 c) a transcriptional and translational termination regulatory
region, wherein the regulatory regions are functional in the
host, and

d) a selection marker gene for selecting transformed host cells;
and

- recovering the starch-binding domain polypeptide.

The invention also relates to a method for optimization of
5 SBD expression in a *Bacillus* host, the method comprising the
steps of:

- a. expression in the host of a SBD fused to a reporter molecule;
- b. monitoring the concentration of expressed SBD in the super-
natant of the fermented host by measuring the intrinsic property
10 or properties of the reporter molecule.

Finally the invention relates to a method of producing a hy-
brid, wherein the hybrid is expressed in a *Bacillus* host, grow-
ing the transformed host under conditions whereby the trans-
formed culture is substantially free of un-transformed cells;
15 incubating the transformed culture in a nutrient medium, whereby
the hybrid is overproduced; and recovering the hybrid.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the sequence alignment (of the D and E-domain)
20 of the maltogenic amylase from *B. stearothermophilus* C599 and *B.*
circulans strain 251 CGTase. Written in "Bold" are residues in-
volved in maltose binding via their side-chain and written in
"Italic" are residues with side-chains involved in maltose bind-
ing in MBS 2. S=sequence no.; 1= The *B. stearothermophilus* C599
25 maltogenic amylase sequence; 2= The *B. circulans* strain 251
CGTase sequence; D=domain.

Figure 2 shows the SDS-PAGE gel of the purified E-domain single
SBD: Lane 1 and 3: Molecular weight standards (from above): 94,
67, 43, 30, 20, 14 kDa; lane 2: single SBD (E-domain).

30

DETAILED DESCRIPTION OF THE INVENTION

The inventors of the present invention found by alignment of
the amino acid sequence (in the D and E domains) of the *Bacillus*

stearothermophilus C599 maltogenic amylase that it had a high identity to CGTases, in particular to a *Bacillus circulans* strain 251 CGTase (Lawson, C.L., Montfort, R.v., Strokopytov, B., Rozeboom, H.J., Kalk, K.H., Vries, G.E.d., Penninga, D.,
5 Dijkhuizen, L. and Dijkstra, B. 1994; J. Mol. Biol. 236, p.590-600).

The identity for the E-domains was found to be 48% and the identity for the D-E-domains 45% using the "align" program based on the Smith-Waterman method and BLOSUM45 table (Henikoff S,
10 Henikoff JG, 1992, Proc. Natl. Acad. Sci. USA89:10915-10919).

The structure of the *B. circulans* strain 251 CGTase in complex with maltose was published by Lawson C.L. et al. (1994), supra) and can be found in Brookhaven data-bank under the code 1CDG.pdb.

15 The CGTase structure consists of five domains of which the A, B and C domains are similar to the domains found in α -amylases. The additional domains named D and E, respectively, are unique for CGTases.

The function of the D-domain is believed to form a stable
20 linkages between the E-domain and the catalytic active domains. In the E-domain two maltose-binding sites, denoted maltose binding site 1 (MBS 1) and maltose binding site 2 (MBS 2) have been identified in the structure. W616 and W662 are stacking with the glucose rings and the side-chains of K651 and N667 are forming
25 hydrogen bonds to -OH groups at the glucose rings in MBS 1.

As indicated in the alignment (see Figure 1) all four residues are also found in the maltogenic amylase. The MBS 2 is defined by stacking with Y633 and H-bonds to the side-chain of residue T598, N603, N627 and Q628. Also in the maltogenic amylase the positions T598, Q628 and Y633 are present, whereas the
30 N603 position is substituted by K (Lys) which is also potential to form H-bond to -OH groups. No analogue to N627 is present.

Based on the alignment and the present of the residues analogue to MBS defining residues in the CGTase it is most likely that the maltose binding sites are also found in the maltogenic amylase. The high homology between the CGTase and the maltogenic amylase indicates the same overall structure, and the structure of the CGTase can therefore be used for determining the different domains in the maltogenic amylase. Using the alignment and the CGTase structure the starting point of the D-domain was determined to amino residue 494 and the E-domain determined to start with amino residue 576. The theoretical sizes of D-E and E-domains are therefore 193 amino acids and 111 amino acids respectively, corresponding to 20 kDa and 12 kDa.

As described above the inventors have found that the maltogenic amylase product produced by *Bacillus stearothermophilus* C599 disclosed in EP patent no. 120,693 (Novo Industri A/S) comprises a SBD in the C-terminal part of the protein sequence.

In the first aspect the invention relates to an isolated DNA sequence comprising a DNA sequence encoding the E domain of the maltogenic amylase produced by *Bacillus stearothermophilus* C599 without having the enzymatic activity of the maltogenic amylase produced by *Bacillus stearothermophilus* C599. The E domain coding DNA sequence is the sequence shown in SEQ ID NO: 1. The corresponding protein sequence is shown in SEQ ID NO: 2.

In an embodiment the isolated DNA sequence of the invention further comprising the D-domain of the maltogenic amylase produced by *Bacillus stearothermophilus* C599 without having the enzymatic activity of the maltogenic amylase produced by *Bacillus stearothermophilus* C599. In other words, the isolated DNA sequence contains the DE-domain coding DNA sequence. The DE-domain coding sequence is shown in SEQ ID NO: 3. The DE domain protein sequence is shown in SEQ ID NO: 4.

In a second embodiment the invention relates to a single SBD with starch-binding affinity encoded by the above-mentioned isolated DNA sequences of the invention.

The single SBD with starch-binding affinity is shown in SEQ ID NO: 2 (E-domain) and SEQ ID NO: 4 (DE-domain).

Example 1 below describes the construction of an expression vector and expression of the single SDB from the maltogenic amylase from *Bacillus stearothermophilus* C599 in a *Bacillus* host.

- Further, in the third aspect the invention relates to a method of producing a single starch-binding domain polypeptide in a *Bacillus* host cell, the method comprising the steps of:
- growing under conditions to overproduce the starch-binding domain, in a nutrient medium, a *Bacillus* host cell which has been transformed with an expression cassette which includes, as operably joined components:
 - a) a transcriptional and translational initiation regulatory region,
 - b) a DNA sequence encoding the starch-binding domain polypeptide,
 - c) a transcriptional and translational termination regulatory region, wherein the regulatory regions are functional in the host, and
 - d) a selection marker gene for selecting transformed host cells;
 - recovering the starch-binding domain polypeptide.

Several SBDs have been expressed in *E. coli*, however, none have been reported to be expressed and secreted from *Bacillus* sp. *E. coli*, as an expression host for heterologous proteins has several advantages over *Bacillus* spp., firstly because *E. coli* has a periplasmic space where proper folding of heterologous expressed genes is possible (for review see for example Hockney, R.C. (1994) TIBTECH, vol.12, p.456-463). Especially the oxidiz-

ing potential and the existence of disulfide oxidoreductases in the periplasma are necessary when expressing proteins with a functionality dependent on properly arranged disulfide bridges (Emmanuel Brun et al. (1995).

5 Furthermore, the periplasma of *E. coli* also acts in protecting the heterologously expressed protein towards the action of proteases present in the supernatant as well as the cytoplasm.

It is also known that when expressing secreted proteins with disulfide bridges in *Bacillus subtilis* the level of expression
10 drops significantly (Bertus van den Berg et al., (1993), Introduction of disulfide bonds into *Bacillus subtilis* neutral protease. Protein Engineering, vol.6 no.5, p. 521-527).

Another problem with heterologue expression is the proteolytic degradation of the expressed protein. *Bacillus subtilis*
15 is known to express at least 7 different extracellular proteases (Eds. A.L. Sonenshein, J.A. Hoch and Richard Losick (1993) *Bacillus subtilis* and other Gram-Positive Bacteria, American Society for microbiology, p.939).

Especially for SBDs, which are highly hydrophobic proteins,
20 the translocation of the protein when expressed in *Bacillus subtilis* could be severely hampered and even cause cell death due to deleterious effects if the protein gets anchored to the cell membrane because of its hydrophobicity.

In the forth aspect the present invention relates to a *Bacillus*
25 *lus* host cell transformed with a vector comprising a DNA sequence coding for a SBD and capable of expressing the sequence.

In a preferred embodiment, the expressed single SBD or SBD-containing polypeptide has a molecular weight (M_w) which is equal to or higher than about 4 kD. Preferably, the M_w is equal
30 to or below about 35 kD, more preferably about 32 kD, even more preferably about 30 kD, especially about 25 kD.

As mentioned above the D-domain of *Bacillus stearothermophilus* C599 has a M_w around 12 kD and the DE-domain a M_w around 20 kD.

The SBD may be expressed in the form of a single SBD as defined above, i.e., a polypeptide comprising one SBD. Alternatively, the SBD may be expressed in the form of a dimer or trimer or even a polymer, i.e. a polypeptide or protein comprising two, three, or even more than three identical SBD "units".

The SBD can also be expressed as a part of a multi-domain polypeptide, the non-SBD part of such a polypeptide being for example one, two or even more domains without catalytic activity.

Most SBDs can be expressed according to the present invention, i.e. by means of a transformed *Bacillus* host. In a preferred embodiment SBDs, obtainable from a microorganism or a plant, more preferably from a bacterium or from a fungus, are expressed.

Examples of SBDs from bacteria and fungus include SBDs obtainable from species mentioned above in the "Background" section, in particular the ones belonging to bacteria genus *Bacillus* and the fungus genus *Aspergillus*.

The *Bacillus* host cell of the invention is a neutralophilic or an alkalophilic or a mesophilic or a thermophilic host cell.

Examples of hosts which are useful in the context of the present invention are hosts from the species *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens*. However, it is contemplated that other *Bacillus* species may also be useful hosts for expression of SBDs.

As described in further detail below, the host cell of the invention is transformed with a vector comprising a SBD encoding DNA sequence. Preferably, the vector is integrated into the

genome of the host, more preferably it has been amplified on the genome.

In another preferred embodiment of the invention, the vector is present as an expression plasmid, preferably as a multi-
5 copy plasmid.

In the fifth aspect the present invention relates to a *Bacillus* expression vector which carries an inserted SBD-encoding DNA sequence. Preferably, the expression cassette of the vector comprises regulatory regions from a *Bacillus* sp., more preferably
10 are such regulatory regions endogenous to the host.

In a sixth aspect, the present invention relates to a method for producing a SBD polypeptide, the method comprising the steps of:

- growing under conditions to overproduce the starch-binding domain,
15 main, in a nutrient medium, *Bacillus* host cells which have been transformed with an expression cassette which includes, as operably joined components,
 - a) a transcriptional and translational initiation regulatory region,
 - 20 b) a DNA sequence encoding the starch-binding domain polypeptide,
 - c) a transcriptional and translational termination regulatory region, wherein the regulatory regions are functional in the host, and
 - 25 d) a selection marker gene for selecting transformed host cell; and
- recovering the starch-binding domain polypeptide.

In its seventh aspect, the present invention relates to a method for optimization of SBD expression in a *Bacillus* host,
30 the method comprising the steps of expression in the host of a SBD fused to a reporter molecule; and monitoring the concentration of expressed SBD in the supernatant of the fermented host

by measuring the intrinsic property or properties of the reporter molecule.

In a preferred embodiment, the reporter molecule is a Green Fluorescent Protein, and the intrinsic property is fluorescence
5 emission.

In its eighth and ninth aspect, the invention relates to a polypeptide hybrid consisting essentially of one or more starch-binding domain(s) fused to a green fluorescent protein, and to a method of producing such a hybrid by expression in a *Bacillus*
10 host, growth of the transformed host under conditions whereby the transformed culture is substantially free of untransformed cells; incubation of the transformed culture in a nutrient medium, whereby the hybrid is overproduced; and recovery of the hybrid.

15

EXPRESSION OF A SINGLE SBD

Recombinant expression vectors

A recombinant vector comprising a DNA construct encoding the
20 single SBD of the invention may be any vector which conveniently may be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell, into which it is to be introduced. This introduction of vector into the host cell is often referred to as the transformation of the host cell.
25 Such transformation indicates introduction of DNA into a host cell by using, e.g. protoplasts, natural competent cells, transfection, conjugation, electroporation, or any equivalent method. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal
30 entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome in part or in its entirety and replicated

together with the chromosome(s), into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the single SBD of the invention is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the SBD.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* α -amylase gene, the *Bacillus amyloliquefaciens* α -amylase gene, the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylosidase gene, or the phage Lambda P_R or P_L promoters or the *E. coli* lac, trp or tac promoters. Alternatively, it is possible to design integration vectors such that the DNA encoding the SBD will only become functionally expressed once it is properly integrated into the host genome, e.g. downstream from a resident promoter.

The DNA sequence encoding the SBD of the invention may also, if necessary, be operably connected to a suitable terminator.

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, or a

gene encoding resistance to e.g. antibiotics like kanamycin, chloramphenicol, erythromycin, tetracycline, spectinomycine, or the like, or resistance to heavy metals or herbicides.

To direct a SBD of the present invention into the secretory
5 pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the SBD in the correct reading frame. Secretory signal sequences are commonly
10 positioned 5' to the DNA sequence encoding the. The secretory signal sequence may be that normally associated with the SBD or may be from a gene encoding another secreted protein.

The procedures used to ligate the DNA sequences coding for the present SBD, the promoter and optionally the terminator
15 and/or secretory signal sequence, respectively, or to assemble these sequences by suitable PCR amplification schemes, and to insert them into suitable vectors containing the information necessary for replication or integration, are well known to persons skilled in the art (cf., for instance, Sambrook et al.,
20 op.cit.).

Green Fluorescent Protein (GFP) has become a widely used reporter molecule for monitoring gene expression, tracers of cell lineage and as fusion tags for proteins.

(Andreas Cramer et al. (1996) Improved Green Fluorescent Protein by molecular evolution using DNA shuffling, Nature Biotechnology, Vol. 14, p. 315-319; Andrew B. Cubitt et al. (1995), Understanding, improving and using fluorescent proteins, TIBS, Vol. 20, p. 448-455.

GFP could be fused to SBD's creating a fusion protein having
30 the starch-binding property as well as the fluorescent properties. The expression of this fusion protein could be used to monitor the expressing of SBD's in *Bacillus* species and hereby be used to optimize expression levels of given SBD's.

EXAMPLES**MATERIALS AND METHODS****Strains:**

- 5 *Bacillus stearothermophilus* C599 (EP 120,683) comprises the maltogenic amylase.

Bacillus subtilis DN1885 and Toc46 Diderichsen et al., (1990), Journal of Bacteriology, Vol. 172, p. 4315-4321)

10 **Plasmids:**

 pDN1981 (P.L. Jørgensen, C.K.Hansen, G.B.Poulsen and B. Diderichsen, (1990), In vivo genetic engineering: homologues re-combination as a tool for plasmid construction, Gene, 96, p. 37-41.)

15

General molecular biology methods:

- DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al., (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for *Bacillus*". John Wiley and Sons, 1990).
- 20

- Enzymes for DNA manipulations were used according to the specifications of the suppliers.
- 25

EXAMPLES**EXAMPLE 1**

30 Construction of an expression vector encoding a single SBD

 Oligonucleotide PCR primers were designed to express the E-domain alone and the D+E domain part of the AmyM protein (*i.e.* maltogenic amylase of *Bacillus stearothermophilus* C599). The ra-

tionale was to add the signal sequence of the *Bacillus licheniformis* α -amylase (AmyL, Termamyl™, see P.L. Jørgensen et al. (1990), Gene, 96, p. 37-41) in front of these AmyM fragments in attempts to have the proteins secreted from *Bacillus*.

5 The following primers were used:

#110755:

PstI

5'-GATG**CCTGCAG**CAGCGGCGTCCGCTTCAGCGCCGC-3' (SEQ ID NO: 5)

(the underscored region corresponds to pos. 1783-1798 in the
10 amyM sequence, Genbank Accession nb. M36539)

#110756:

PstI

5'-GATG**CCTGCAG**CAGCGGCGAGTGGAAACGCAGACATCG-3' (SEQ ID NO: 6)

15 (the underscored region corresponds to pos. 2032-2049 in the
amyM sequence, Genbank Accession nb. M36539)

#110757:

EcoRI BamHI

20 5'-GATG**GAATTCGGATCC**TCCATATGTACTACTCC-3' (SEQ ID NO: 7)

(the underscored region corresponds to pos. 2569-2553 in the
amyM sequence, Genbank Accession nb. M36539)

Template for the PCR reaction was a sample of plasmid
pDN1413. This is essentially plasmid pUB110 containing the amyM
25 gene fragment, derived from the deposited strain NCIB 11837 via
plasmid pDN452 which is described in EP 120,693.

Conditions for the PCR amplification were the following:
94°C for 2 minutes, then 20 cycles of 49°C for 30 seconds, 43°C
for 1 minute, 72°C for 2 minutes, then one cycle of 72°C for 5
30 minutes.

Correctly sized PCR fragments were obtained upon amplification. Primer #110755 together with #110757 resulted in a 820

basepair fragment, primer #110756 together with #110757 resulted in a 571 basepair fragment.

PCR fragments were purified using a QIAquick PCR Purification Kit Cat. No. 28106 from Qiagen, and digested with EcoRI + PstI.

5 Plasmid pDN1981 (P.L. Jørgensen et al. (1990), Gene, 96, p. 37-41) was used as cloning vector. pDN 1981 was digested with EcoRI + PstI, and the 3.9 kb fragment purified from an agarose gel. The vector fragment was ligated with each of the digested PCR fragments, and the ligation mixtures transformed into compe-
10 tent cells of *Bacillus subtilis* DN1885 (Diderichsen et al., Journal of Bacteriology, vol. 172, p. 4315-4321, 1990), selecting kanamycin resistance (10 µg/ml). Four colonies from each transformation were reisolated and grown in liquid TY cultures for plasmid preparation. The extracted plasmids all had the correct
15 structure, as judged by restriction digests.

Two transformants of each kind were kept:

SJ4302 and SJ4303 both contained plasmids harboring the #110755 + #110757 PCR fragment, i.e. encoding the D+E domain.

SJ4304 and SJ4305 both contained plasmids harboring the #110756
20 + #110757 PCR fragment, i.e. encoding the E-domain only.

Expression of domains:

Strains SJ4302-SJ4305 were inoculated in 10 ml TY broth containing 0.4% glucose and 10µg/ml kanamycin, and incubated at
25 37°C with shaking for two days.

Strain DN1885 (the *B. subtilis* host strain) was inoculated in 10 ml TY broth with 0.4% glucose, and incubated at 37°C with shaking for two days.

Supernatants were analyzed by SDS-Polyacrylamide gel electro-
30 phoresis.

In the supernatant from strain SJ4302, a protein with an apparent molecular weight of approximately 25 kDa was observed. It

was less obvious that SJ4303 produced a similar protein. This protein was not seen in the other 3 samples.

The difference between SJ4302 and SJ4303 may be due to these clones harboring PCR amplified constructs, that were not verified by DNA sequencing - an error might thus have been introduced into the SJ4303 clone.

In the supernatant from strains SJ4304 and SJ4305, a protein with an apparent molecular weight of approximately 10 kDa was observed. This protein was not observed in the other three samples.

In conclusion, a protein as expected was produced from the D+E domain clone SJ4302, and a protein as expected was produced from the E-domain clones SJ4304-SJ4305.

No difference in expression level (amount of accumulated domain) was observed when the strains were simply propagated as above, or when the strains were propagated in broth as above, but supplemented with the protease inhibitor Complete from Boehringer Mannheim (CompleteTM Protease inhibitor cocktail tablets Cat. No. 1697498; One tablet was dissolved in 2 ml water, and 160 microliters of this solution added to each 10 ml culture). This concentration of protease inhibitor allowed growth, but almost totally inhibited the extracellular proteases present in the DN1885 broth, as judged from spotting broth on agar plates with casein.

EXAMPLE 2

Expression and purification of single SBD (E-domain).

Expression of the E-domain

PSJ4305 (E-domain clone) was transformed into competent cells of *B. subtilis* ToC46 (Diderichsen et al., Journal of Bacteriology, vol. 172, p. 4315-4321, 1990), selecting kanamycin resistance (10 mg/ml), and a transformant was kept as SJ4547.

50 shake flasks of 500 ml total volume, each containing 200 ml of TY broth supplemented with 0.4% glucose and 10 mg/ml kanamycin, were inoculated with SJ4547 and incubated with shaking at 300 rpm at 37°C for 45 hours, and the supernatant separated from the cells by centrifugation.

Purification of the E-domain

The supernatant was centrifuged (4500 rpm, 15 min, 8°C) using a Sorvall RC-3B centrifuge, equipped with a 4600 A rotor head, followed by subsequent filtration through a 0.7 mm glass microfibre filter. The supernatant was applied to α -cyclodextrin-agarose (1.6 x 5 cm) in 25 mM sodium acetate, 1 mM CaCl_2 , 0.5 M NaCl, pH 5.0, at a flow rate of 300 ml h^{-1} . The column was washed (10 column volumes) using 25 mM sodium acetate, 1 mM CaCl_2 , 0.5 M NaCl, pH 5.0, and the single SBD was eluted in the same buffer containing 2% (w/v) α -cyclodextrin. The eluted SBD was pooled and dialyzed against 50 mM sodium acetate, 1 mM CaCl_2 , pH 5.0. The purified single SBD was homogeneous as determined using SDS-PAGE (see below). N-terminal sequencing showed SGTQTSVVF and confirmed that the purified E-domain is identical to residue Ser576 of the maltogenic amylase of *Bacillus stearothermophilus* C599.

Approximately 21 mg homogeneous SBD was purified from 1 L culture filtrate in ten subsequent runs on α -cyclodextrin-agarose. SDS-PAGE analysis of the purified SBD is shown in Figure 2.

Example 3

Starch binding of single SBD

Adsorption of single SBD onto granular starch is determined by incubating increasing amounts of SBD (0-3 mg/ml) with granular corn starch (10 mg/ml) in 5 mM sodium acetate, pH 3.6 at 4°C for 16 hours, essentially as described (Belshaw & Wil-

liamson, 1990). The reaction is terminated by centrifugation and the protein concentration in the supernatant is subsequently determined and subtracted from the total protein to give the amount of starch bound protein.

CLAIMS

1. An isolated DNA sequence comprising a DNA sequence encoding the E-domain of the maltogenic amylase produced by *Bacillus stearothermophilus* C599 without having the enzymatic activity of the maltogenic amylase produced by *Bacillus stearothermophilus* C599.
5
2. The isolated DNA sequence according to claims 1, wherein the E domain sequence is the sequence shown in SEQ ID NO: 1.
10
3. The isolated DNA sequence according to claims 1 or 2 further comprising the D-domain of the maltogenic amylase produced by *Bacillus stearothermophilus* C599 without having the enzymatic activity of the maltogenic amylase produced by *Bacillus stearothermophilus* C599.
15
4. The isolated DNA sequence according to claims 3, wherein the D+E domain sequence is the sequence shown in SEQ ID NO: 3.
- 20 5. A single SBD with starch-binding affinity encoded by a DNA sequence according to any of claims 1 to 4.
6. The single SBD according to claim 5 having the sequence shown in SEQ ID NO: 2 or SEQ ID NO: 4.
25
7. A *Bacillus* host cell transformed with a vector comprising a DNA sequence coding for a single starch-binding domain and which is capable of expressing said sequence.
- 30 8. The host cell according to claim 7, wherein the DNA sequence is of another origin than *Bacillus* ssp.

9. The host cell according to any of claims 7 or 8 which is capable of expressing the starch-binding domain as a single polypeptide domain.
- 5 10. The host cell according to any of the claims 7-9, wherein the vector comprises a DNA sequence encoding for a single starch-binding domain.
- 10 11. The host cell according to any of the claims 7-10, wherein the vector comprises a DNA sequence encoding for a starch-binding domain which is linked to at least one other non-catalytically active domain.
- 15 12. The host cell according to any of the claims 7-11, wherein the starch-binding domain is obtainable from a microorganism or from a plant, preferably a bacterium or a fungus.
13. The host cell according to claim 12, wherein the bacterium is selected from the group consisting of the genus *Bacillus*.
- 20 14. The host cell according to claim 13, wherein the fungus is selected from the group consisting of the genus *Aspergillus*.
15. The *Bacillus* host cell according to any of the claims 7-14
25 which is neutralophilic, alkalophilic, mesophilic or thermophilic.
16. The *Bacillus* host cell according to claim 15 which is selected from the group comprising the following species: *Bacillus*
30 *subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens*.

17. The host cell according to any of the claims 7-16, wherein the vector is integrated into the genome of the untransformed host.

5 18. The host cell according to any of the claims 7-17, wherein the vector is present as an expression plasmid.

19. The host cell according to any of the claims 7-18, wherein the vector has been amplified on the genome or the expression
10 plasmid is a multicopy plasmid.

20. A *Bacillus* expression vector which carries an inserted DNA sequence encoding a single starch-binding domain.

15 21. The vector according to claim 20 in which the expression cassette comprises regulatory regions from a *Bacillus* sp.

22. The vector according to claim 21, wherein the *Bacillus* sp. regulatory regions are endogeneous to the host.

20

23. A method for producing a single starch-binding domain polypeptide in a *Bacillus* host cell, the method comprising the steps of

■ growing under conditions to overproduce starch-binding domain
25 in a nutrient medium, a *Bacillus* host cell which has been transformed with an expression cassette which includes, as operably joined components,

a) a transcriptional and translational initiation regulatory region,

30 b) a DNA sequence encoding the starch-binding domain polypeptide,

- c) a transcriptional and translational termination regulatory region, wherein the regulatory regions are functional in the host, and
- d) a selection marker gene for selecting transformed host cells;
- 5 and
- recovering the starch-binding domain polypeptide.

24. A method for optimization of SBD expression in a *Bacillus* host, the method comprising the steps of

- 10 a. expression in the host of a SBD fused to a reporter molecule;
- b. monitoring the concentration of expressed SBD in the supernatant of the fermented host by measuring the intrinsic property or properties of the reporter molecule.

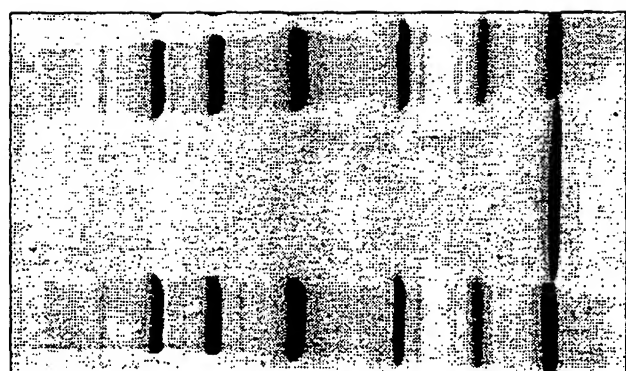
- 15 25. The method according to claim 24, wherein the reporter molecule is a Green Fluorescent Protein, and the intrinsic property is fluorescence emission.

- 26. A method of producing the hybrid according to claim 24 or
- 20 25, wherein the hybrid is expressed in a *Bacillus* host, growing the transformed host under conditions whereby the transformed culture is substantially free of un-transformed cells; incubating the transformed culture in a nutrient medium, whereby the hybrid is overproduced; and recovering the hybrid.

	S	470	480	490	500	510	520
5	1	ISV-SNGSVASFTLAPGAVSVWQYSTSASAPQIGSVAPNMGIPGNVVTIDGKFGTTQGT					
	2	LSVGSGGAASNFTLAAGGTAVWQYTAATATPTIGHVGPMMAKPGVTITIDGRGFGSSKGT					
	D	CC					
10	S	530	540	550	560	570	580
	1	VTFGGVAT---VKSWTSNRIEVYVVPNMAAGLTDVKVTAGGVSSNL-Y-SYNILSGTQTS					
	2	VYFGTTAVSGADITSWEDTQIKVIPAVAGGNYNIKVANAAGTASNVDNFEVLSGDQVS					
	D	DD					
	S	590	600	610	620	630	640
15	1	VVFTVKSAPP T NLGDKiYLtGNIPElGN W STdTSgAVNNAQgPLlAPNYPdWfYVfSVPA					
	2	VRE-VVNNA T ALGQ N YLtGSVSELGN W DPaKaIGPMY NQ ---VvYQYPnWYyDVSvPA					
	D	EE					
	S	650	660	670	680		
20	1	GKtIQ K FFIKRADGTIQ W ENGs N HvATTPTGATGNITvTWQN					
	2	GKtIEF K FLKKQGS-TVt W EGGS N HtFTAPSSGTATINvNWQP					
	D	EE					

Fig. 1

2/2



lane 1 lane 2 lane 3

Lane 1 and 3: Molecular weight standards (from above): 94, 67,
43, 30, 20, 14 kDa
Lane 2: single SBD (E-domain)

Fig. 2

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S
 (B) STREET: Novo Alle
 (C) CITY: Bagsvaerd
 (E) COUNTRY: Denmark
 (F) POSTAL CODE (ZIP): DK 2880
 (G) TELEPHONE: +45 4444 8888
 (H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: Expression of SBDs

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 327 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "E-domain"

(vi) ORIGINAL SOURCE:

(B) STRAIN: B. steatothermophilus C599

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1..327

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGT GGA ACG CAG ACA TCG GTT GTG TTT ACT GTG AAA AGT GCG CCT CCG	48
Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala Pro Pro	
1 5 10 15	
ACC AAC CTG GGG GAT AAG ATT TAC CTG ACG GGC AAC ATA CCG GAA TTG	96
Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro Glu Leu	
20 25 30	
GGG AAT TGG AGC ACG GAT ACG AGC GGA GCC GTT AAC AAT GCG CAA GGG	144
Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala Gln Gly	
35 40 45	
CCC CTG CTC GCG CCC AAT TAT CCG GAT TGG TTT TAT GTA TTC AGC GTT	192
Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe Ser Val	
50 55 60	
CCA GCA GGA AAG ACG ATT CAA TTC AAG TTC ATC AAG CGT GCG GAT	240
Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg Ala Asp	
65 70 75 80	
GGA ACG ATT CAA TGG GAG AAT GGT TCG AAC CAC GTG GCC ACA ACT CCC	288
Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr Thr Pro	
85 90 95	

ACG GGT GCA ACC GGT AAC ATT ACT GTT ACG TGG CAA AAC
 Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn
 100 105

327

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 109 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala Pro Pro
 1 5 10 15
 Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro Glu Leu
 20 25 30
 Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala Gln Gly
 35 40 45
 Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe Ser Val
 50 55 60
 Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg Ala Asp
 65 70 75 80
 Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr Thr Pro
 85 90 95
 Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 576 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "DE domain"

(vi) ORIGINAL SOURCE:

(B) STRAIN: B. stearothermophilus C599

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..576

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCC GCT TCA GCG CCG CAA ATC GGA TCG GTT GCT CCA AAT ATG GGG ATT
 Ser Ala Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile
 1 5 10 15
 CCG GGT AAT GTG GTC ACG ATC GAC GGG AAA GGT TTT GGG ACG ACG CAG
 Pro Gly Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln
 20 25 30

48

96

GGA	ACC	GTG	ACA	TTT	GGC	GGA	GTG	ACA	GCG	ACT	GTG	AAA	TCC	TGG	ACA	144
Gly	Thr	Val	Thr	Phe	Gly	Gly	Val	Thr	Ala	Thr	Val	Lys	Ser	Trp	Thr	
		35					40					45				
TCC	AAT	CGG	ATT	GAA	GTG	TAC	GTT	CCC	AAC	ATG	GCC	GCC	GGG	CTG	ACC	192
Ser	Asn	Arg	Ile	Glu	Val	Tyr	Val	Pro	Asn	Met	Ala	Ala	Gly	Leu	Thr	
		50				55					60					
GAT	GTG	AAA	GTC	ACC	GCG	GGT	GGA	GTT	TCC	AGC	AAT	CTG	TAT	TCT	TAC	240
Asp	Val	Lys	Val	Thr	Ala	Gly	Gly	Val	Ser	Ser	Asn	Leu	Tyr	Ser	Tyr	
		65				70					75				80	
AAT	ATT	TTG	AGT	GGA	ACG	CAG	ACA	TCG	GTT	GTG	TTT	ACT	GTG	AAA	AGT	288
Asn	Ile	Leu	Ser	Gly	Thr	Gln	Thr	Ser	Val	Val	Phe	Thr	Val	Lys	Ser	
				85					90					95		
GCG	CCT	CCG	ACC	AAC	CTG	GGG	GAT	AAG	ATT	TAC	CTG	ACG	GGC	AAC	ATA	336
Ala	Pro	Pro	Thr	Asn	Leu	Gly	Asp	Lys	Ile	Tyr	Leu	Thr	Gly	Asn	Ile	
			100					105					110			
CCG	GAA	TTG	GGG	AAT	TGG	AGC	ACG	GAT	ACG	AGC	GGA	GCC	GTT	AAC	AAT	384
Pro	Glu	Leu	Gly	Asn	Trp	Ser	Thr	Asp	Thr	Ser	Gly	Ala	Val	Asn	Asn	
		115					120					125				
GCG	CAA	GGG	CCC	CTG	CTC	GCG	CCC	AAT	TAT	CCG	GAT	TGG	TTT	TAT	GTA	432
Ala	Gln	Gly	Pro	Leu	Leu	Ala	Pro	Asn	Tyr	Pro	Asp	Trp	Phe	Tyr	Val	
		130				135					140					
TTC	AGC	GTT	CCA	GCA	GGA	AAG	ACG	ATT	CAA	TTC	AAG	TTC	TTC	ATC	AAG	480
Phe	Ser	Val	Pro	Ala	Gly	Lys	Thr	Ile	Gln	Phe	Lys	Phe	Phe	Ile	Lys	
		145				150					155				160	
CGT	GCG	GAT	GGA	ACG	ATT	CAA	TGG	GAG	AAT	GGT	TCG	AAC	CAC	GTG	GCC	528
Arg	Ala	Asp	Gly	Thr	Ile	Gln	Trp	Glu	Asn	Gly	Ser	Asn	His	Val	Ala	
				165					170					175		
ACA	ACT	CCC	ACG	GGT	GCA	ACC	GGT	AAC	ATT	ACT	GTT	ACG	TGG	CAA	AAC	576
Thr	Thr	Pro	Thr	Gly	Ala	Thr	Gly	Asn	Ile	Thr	Val	Thr	Trp	Gln	Asn	
			180					185					190			

(i) SEQUENCE CHARACTERISTICS:

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gly Thr Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr
35 40 45

4

Ser Asn Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr
 50 55 60

Asp Val Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr
 65 70 75 80

Asn Ile Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser
 85 90 95

Ala Pro Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile
 100 105 110

Pro Glu Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn
 115 120 125

Ala Gln Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val
 130 135 140

Phe Ser Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys
 145 150 155 160

Arg Ala Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala
 165 170 175

Thr Thr Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn
 180 185 190

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer #110755"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATGCTGCAG CAGCGGCGTC CGCTTCAGCG CCGC

34

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer #110756"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GATGCTGCAG CAGCGGCGAG TGAACGCAG ACATCG

36

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer #110757"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GATGGAATTC GGATCCTCCA TATGTACTAC TCC

33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00411

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Medline, accession no. 96067699; & Erur J Biochem, vol 233, häfte 2, Oct 1995, Le Gal-Coeffet MF et al: "Expression in Aspergillus niger of the starch-binding domain of glucoamylase. Comparison with the proteolytically produced starch- binding domain", page 561 - page 567 --	1-26
X	Database Medline, accession no. 97115811; & J Biol Chem vol. 271, häfte 51, 20 Dec 1996, Penninga D, et al: "The raw starch binding domain of cyclodextrin glycosyltransferase from Bacillus circulans strain 251", page 32777 - page 32784 --	1-26

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 January 1999

Date of mailing of the international search report

15 -01- 1999

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 98/00411

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Pir database, accession no. S28784, Diderichsen, B. et al: "Cloning of a maltogenic alpha-amylase from Bacillus stearothermophilus"; & FEMS Microbiol. Lett. 56, 30-60, 1998 --	1-26
A	WO 9816190 A1 (NOVO NORDISK A/S), 23 April 1998 (23.04.98) --	1-26
A	EP 0120693 A1 (NOVO INDUSTRI A/S), 3 October 1984 (03.10.84) -- -----	1-26

INTERNATIONAL SEARCH REPORT
Information on patent family members

01/12/98

International application No.

PCT/DK 98/00411

Patent document cited in search report			Publication date	Patent family member(s)	Publication date
WO	9816190	A1	23/04/98	AU 4550897 A	11/05/98
EP	0120693	A1	03/10/84	SE 0120693 T3	
				CA 1214407 A	25/11/86
				DK 135983 D	00/00/00
				DK 153799 B,C	05/09/88
				DK 161084 A	26/09/84
				JP 1786423 C	10/09/93
				JP 4072505 B	18/11/92
				JP 60002185 A	08/01/85
				US 4598048 A	01/07/86
				US 4604355 A	05/08/86

Daniel Almont

Fr m: Holly Pekowsky
Sent: Wednesday, May 15, 2002 3:33 PM
To: Daniel Almont
Subject: RE: Name Search Request for Spring 2003 Sportswear Shirts

do you have a copy?

-----Original Message-----

From: Daniel Almont
Sent: Wednesday, May 15, 2002 3:32 PM
To: Holly Pekowsky
Subject: RE: Name Search Request for Spring 2003 Sportswear Shirts

not long after the initial request, 5/3, although you did send a follow-up on 5/7. So probably shortly after that.

-----Original Message-----

From: Holly Pekowsky
Sent: Wednesday, May 15, 2002 3:16 PM
To: Daniel Almont
Subject: RE: Name Search Request for Spring 2003 Sportswear Shirts

yes. When do you think you did them?

-----Original Message-----

From: Daniel Almont
Sent: Wednesday, May 15, 2002 3:11 PM
To: Holly Pekowsky
Subject: RE: Name Search Request for Spring 2003 Sportswear Shirts

I'm thinking that I did do these. Are you saving a copy set of everything that we do?

We definitely should. In the set that I did yesterday, I recognized a couple of marks that I searched in the last week or two. I just redid them. Hopefully we came to the same conclusion both times.

> -----Original Message-----

> **From:** Dilliplane, Marci
> **Sent:** Friday, May 03, 2002 11:18 AM
> **To:** Brooks, Ellen
> **Subject:** Name Search Request for Spring 2003 Sportswear Shirts
>
> HI ELLEN,
>
> I NEED TO GET THE FOLLOWING MODEL NAMES LEGALLY APPROVED FOR SP03
> SPORTSWEAR SHIRTS:
>
> CARLYLE
> WILLIAM
> VINTAGE BUTTONDOWN
> VINTAGE BAND
> VINTAGE MARLOWE
>
> THANKS!!!!
> MARCI